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The Effects of Skimmed Milk, Soybean Flour and Sardine Fish Powder on Osteoporotic Female Rats

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Abstract: This study was carried out to investigate the effects of skimmed milk, soybean flour and whole dried sardine fish on osteoporotic female rats. Fifty six female albino rats were divided into two main groups. The first group (10 rats) fed basal diet and maintained as negative control group. The second main group (forty six) was injected with 2mg dexamethsone sodium phosphate daily (synthetic corticosteroid) for 5 weeks, after that the second main group divided into seven groups. Group1 osteoporotic group fed basal diet, meanwhile groups from 2 to 7 osteoporotic rats fed basal diet supplemented with 10% and 20% skimmed milk, soy flour and dried sardine fish. The results indicated that the injection with cortisone caused osteoporosis of rats. Induced osteoporosis caused decreases in body weight gain, serum ostocalcin, Estradiol (E2), calcium (Ca) and phosphorous (P) concentration in serum and femur bone, Boon mineral density (BMD), Boon mineral concentration (BMC) and increases in weight of liver and Parathyroid hormone (PTH) compared to negative control group (healthy rat). However, supplementation diet of osteoporotic rat with 20% skimmed milk, soybean flour and dried sardine fish lead to increases in body weight gain, serum ostocalcin, E2, Ca, P in serum and femur, BMD, BMC and decreases in weight of liver and PTH. The best results were observed of rats fed diet supplemented with 20% skimmed milk. Liver function (AST, ALT and ALP) of osteoporotic rats revealed significantly increase compared to negative control group. The levels of AST, ALT and ALP decreased gradually with increasing sardine fish and soybean flour concentration. Positive control group (osteoporotic rats) showed changes in liver and bone histological structure compared to negative control group. In conclusion, administration diet supplemented with skimmed milk preventing and treated osteoporosis disease more than diet supplemented sardine fish and soybean flour.

Key words: Osteoporosis · Induced osteoporosis · Bone marker · Bone mineral density · Soybean flour · Skimmed milk · Sardine fish

INTRODUCTION

Osteoporosis is considered a major public health problem and it is characterized by decrease bone density, resulting in skeletal fragility and fractures [1-3]. Osteoporosis is a major complication in patient who requires chronic glucocorticoid treatment [4]. Osteoporosis is three times more common in women than in men, partly because of the hormonal changes that occur at the menopause [5]. Osteoporosis also has negatively affects the quality of life of elderly patients. Ten percent of women 55-64% years old and 22.5% of women 65 years and alder reported that osteoporosis interfered with their daily activities [6]. This disease is characterized by a reduction in bone mass and microartichitectaral deterioration of bone tissue, resulting in skeletal fragility and susceptibility to fractures [7, 8]. In the United State, an estimated 44 million Americans or 55 % of people 50 years and alder are both osteoporosis and low bone mass [9]. Osteoporosis affects more than 75 million people in Europe, Japan and USA and causes more than 2.3 million fractures annually in Europe and USA alone. Direct medical expendititures on osteoporotic fractures were estimated at US $ 13.8 billion in 1999 [5]. Prevalence of osteoporosis among adults in Egypt is 14.9% and 12.6% for males and females respectively.

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Osteoporosis increases gradually by age to reach 21.9% age groups 40-<50 years old. Relative osteoporosis among male adolescents is 16.7% and among female adolescent is 0.9% [10]. Osteoporosis is influenced by diet, adequate nutrition, especially calcium intake, plays a major role in the prevention and treatment of osteoporosis [11-13]. Calcium intake influences peak bone mass achieved in early adulthood by influencing skeletal role in preventing bone loss and osteoporotic fractures in later life. Low calcium intake is widespread problem across countries [14]. Numerous studies have shown calcium intake is associated with bone mineral density [15-17].Dairy products are good sources of calcium but plant calcium may be also important in populations that do not consume a large amount of milk [18, 19]. Dried skimmed milk is made by removing fat and almost all moisture from milk followed by pulverization. Dried skimmed milk is more than 96% milk solid, 4% or less moisture and has little fat (about 0.6%). It is about 36% good quality protein and about 51% lactose and is high in vitamin B complex, especially riboflavin. In addition it is about good quality minerals, mainly calcium [20]. Soybean is a species of legume which originated from Asia and was introduced to the USA in 1765 by Samuel Bowen [21]. Soybean products are common dietary sources of protein and calcium and containing high concentration of isoflavones with potential health enhancing properties, such as easing the symptoms of postmenopausal women [22-27] and preventing cardiovascular disease and antimutagenic effects [28]. Soybean products have many health benefits including prevention of breast cancer, prostate cancer and osteoporosis [27,29,30]. Soybean flour have higher concentration from isoflavones (68.27 and 112.25 mg/100g) total Daidzein and total Genistein more than soy protein and tofu [31].

Diet is on equally important factor in bone health. Fish are very beneficial for bone health. Anchovies, caviar, herring, salmon, sardine with bone are high in usable calcium [32]. Small fish with bone may be an important source of calcium in human diet [33]. Fish such as salmon and sardines that include bones is considered nondairy good sources of calcium [18, 34]. Sardine bones are rich in calcium and will be utilized for the production of calcium powder to treat osteoporosis [35].

The objective of this study was to investigate the effect of skimmed milk, soybean flour and whole sardine dried fish (sardine with bone) on osteoporosis in female rats.

MATERIALS AND METHODS

Materials: Dexamethsone sodium phosphate (synthetic corticosteroid) ampoules were obtained from pharmacy Cairo, Egypt. Skimmed soybean flour was obtained from Food Technology Research Institute, Agricultural Research Center, Giza, Egypt. Skimmed milk powder and Sardine fish were purchased from local market, Cairo, Egypt. Kits for blood was purchased from Biodiagnostic Company, Cairo, Egypt. Kits for bone marker was purchased from Sigma Aldrich Chemise GmbH, Germany.

Methods:

Preparation of Sardine Fish Powder: Fresh sardine fish (without head) was cleaning and washing, after that cooking (sardine include bone) with pressure cooked for 30 minutes, then dry in oven at 50°C for 4 hours and milling for powder.

Determination of Protein, Calcium and Phosphorus in Materials: Protein, calcium and phosphorus were determined in skimmed milk, soybean flour and sardine fish powder by the method of AOAC [36].

Determination of Fatty Acid in Dried Sardine Fish: Fatty acid analysis was determined using the method of Bligh and Dyer [37]. Fatty acid was converted to fatty acid methyl ester according to the method described by Gibson and Kneebone [38]. Trace GC Mass Ultra thermo was used to separate fatty acids on 10m capillary column (0.1nm IDx0.2μm). The carrier gas used was helium set a flaw rate of 0.5ml/min and split less, time 1min. The injection temperature was 250°C and FID detector was 350°C. Initial oven temperature was 40°C and rose to 230°C and holding for 20min. Identification of compounds was based on molecular weight of fatty acids.

Experimental Design: Fifty six female albino rats weighting 160±5 g were housed individually in ventilated cages under controlled condition at constant temperature (22°C) and lighting (12h. light - dark cycle) and given free access to food and water at all time. The rats were divided into two main groups. The first group (10 rats) fed on basal diet and served as a control negative group. Basal diet preparation according to Reeves *et al.* [39]. The second group (forty six rats) fed on basal diet and injected with 2mg dexamethsone sodium phosphate (synthetic corticosteroid) to induce osteoporosis for

5 weeks. After that 4 rats were taken from each group to make sure incidence of osteoporosis, then the second control group (forty two rats) divided into seven group 6 each as follows:

Group 1: Osteoporotic rats fed on basal diet preserved as positive control group.

Group 2: Osteoporotic rats fed on basal diet containing 10% skimmed milk.

Group 3: Osteoporotic rats fed on basal diet containing 20% skimmed milk.

Group 4: Osteoporotic rats fed on basal diet containing 10% soybean flour.

Group 5: Osteoporotic rats fed on basal diet containing 20% soybean flour.

Group 6: Osteoporotic rats fed on basal diet containing 10% sardine fish powder.

Group 7: Osteoporotic rats fed on basal diet containing 20% sardine fish powder.

Each rat has been weighted at the beginning, weekly and the end for experimental and food intake was daily recorded. At the end of experimental period (eight weeks), rats were sacrificed after overnight fasting. The blood of each rat was collected and centrifuged at 300 rpm for 20 minutes to obtain the serum, which kept at -20°C until analysis and right femurs were harvested. Each right femur bone was carefully cleaned and the weight was recorded and then stored in formalin buffer 10% formalin until analysis.

Biochemical Analysis:

Bone Marker: Serum osteocalcin was determined according to the method described by Coleman *et al.* [40]. Serum parathyroid hormone (PTH) was determined according the method described by Goltaman *et al*. [41]. Serum estradiol E2 was determined according to the method by Ratcliffe *et al.* [42].

Determination of Calcium and Phosphorus in Serum: Serum calcium and phosphorus were estimated to the method described by Baginsk *et al.* [43] and Patricia [44].

Determination of Calcium and Phosphorus in Bone: Calcium and phosphorus in bone were determined in left femur by colorimetric method according to the method described by Ginder and King [45] and Goldenberg [46].

Determination of Liver Enzymes: ALT and AST were determined by the method of Reitman and Frankal [47]. Serum alkaline phosphate ALP was determined by Rosalki *et al.* [48].

Measurement of Bone Mineral Density and Bone Mineral Concentration: Right femur bone were separated after scarification and femur was weighted, after that bone mineral density BMD and bone mineral concentration BMC of each femur were measured according to the method by Marie-Pierre *et al.* [49], by Dual Energy X-ray Absorption (DEXA) using Norland X-R46, version 3.9.6, Peachtree City, GA, USA) equipped with dedicated software for small animal measurements. This technique provided integrated measurements. These measurements by DEXA bone scanner yielded total, cortical and trabecular BMD and BMC.

Bone and Liver Histology: Right femurs bone and liver were fixed with 10% formalin until analysis. The bone was decalcified with 10% mixture of formic acid and hydrochloric acid solution for 45 day. The rotten tissue processing for light microscopy was performed and tissues were bedded in paraffin.

Statistical Analysis: The results obtained were analyzed using SPSS program (version 18.0) and expressed as mean and standard deviations (SD). Statistical significance ($p<0.05$) among the groups were determined by one-way ANOVA followed by Duncan's multiple range test according to Bailey [50].

RESULTS AND DISCUSSION

Data in Table 1 shows protein percentage, calcium and phosphorus concentration in skimmed milk, soybean flour and dried sardine fish. Results showed that sardine fish content that highest protein percentage (77.4%), skimmed milk content the lowest protein percentage (32%), while soybean flour content 49.8% protein. These results are on line with the results obtained by Mohamed [31], who reported that, the soybean flour content 49.8% protein. Also, Codex [51] reported skimmed milk content 34% protein. Also, data in Table 1 revealed that skimmed milk content the highest calcium concentration (2000 mg/100g) while, soybean flour content the lowest calcium concentration (560 mg/100g) meanwhile, sardine fish content higher calcium concentration (1940 mg/100g). The phosphorus concentration in sardine fish was 1560 mg/100g followed by skimmed milk (1000 mg/100g) and soybean flour (700 mg/100g). These results are in agreement with those obtained Kris-Etherton *et al.* [52],who mentioned that sardines are high in major minerals such phosphorus, calcium and potassium.

Omega-3 fatty acid constituent (ALA, EPA and DHA) represent the major proportion of total fatty acids of sardine fish (35.86%) (Table 2). In general the long chain fatty acid of omega-3 EPA was present in lower concentration (9.78%) than DHA (25.33%). This result is in agreement with those obtained by Soltan [53], who reported omega-3 fatty acid was the major fatty acid in sardine fish.

Data presented in Table 3 demonstrated that the body weight of experimental rats at the beginning showed no significant difference. Meanwhile, showed significant decrease P<0.05 in final body weight (190.4 g), body weight gain (27.8 g) and feed intake (11.58 g) of control positive (suffering from osteoporosis) compared to control negative (healthy rat) (327.4, 163.6 and 15.36 g), respectively. These results are in agreement with the findings of Oliveira [54], who reported that glucocorticoids induced a significant lower weight gain (-9.7%) compared to control group. Meanwhile, rat's administration diet supplemented with 20% skimmed milk and 20% sardine fish recorded significant increase in final body weight and body weight gain (212.40, 44.8 and 207, 44.2 g) compared to positive control group (190.40 and 27.8 g). These results are accordance with the results obtained by Gouda [55], who reported that control positive (osteoporotic rats) showed significant decrease

Table 1: Protein, calcium and phosphorus of skimmed milk, soybean flour and dried sardine fish.

Samples	Protein (%)	Calcium (mg/100g)	Phosphorus (mg/100g)
Skimmed milk	32	2000	1000
Soybean Flour	49.8	560	700
Dried sardine fish	77.4	1940	1560

Table 2: Relative percentage of fatty acid composition in dried sardine fish

	°Total SFA			Omega-6		Omega-3			
	--------------------------------		§Total MUFA	--------------------------------		--			
Sample	Myristic acid	Palmitic acid	Oleic acid	*LA 18:3n-6	**AA 20:4n-6	†ALA 18;3n-3	°EPA 20:5n-3	‡DHA 20:6n-3	Total omega-3
Sardine fish	12.55	23.44	24.93	2.44	0.78	0.75	9.78	25.23	35.86

°Total saturated fatty acid, §Total Mono unsaturated fatty acid, *Linoleic acid, † Linolenic acid, oEicospentaenoic acid, ‡Docosahexaenoic acid **Arachidonic

Table 3: Effect of skimmed milk, soybean flour and dried sardine fish on body weight gain, feed intake and feed efficiency ratio of osteoporotic female rats

Groups	oIBW (g)	°FBW (g)	BWG (g)*	†Feed intake (g)	‡Feed efficiency Ratio
Control negative	163.8±4.14^{a}	327.40±5.17 ^{a}	163.6±7.16 ^{a}	15.36±0.99 ^{a}	0.190±0.78 ^{a}
Control positive	163.20±7.79^{a}	190.40 ±11.17 ^{d}	27.8±6.76 ^{cd}	11.58±0.59 ^{d}	0.043±0.63 ^{b}
10% Skimmed milk	164.80±4.60 ^{a}	203.40±6.07 ^{bcd}	38.6±5.37 ^{bcd}	13.20±0.14 ^{bc}	0.052±0.43 ^{b}
20% Skimmed milk	167.60±8.05 ^{a}	212.40±7.70 ^{b}	44.8±12.23 ^{b}	13.94±0.94 ^{b}	0.057±0.76 ^{b}
10% Soybean flour	163.0±2.00^{a}	192.8±5.97$^{c\ d}$	29.8±0.59 ^{cd}	12.90±0.27 ^{c}	0.041±0.63 ^{b}
20% Soybean flour	165.60±3.49 ^{a}	198.20±14.11 ^{bcd}	32.4±12.34 ^{bcd}	13.28±0.46 ^{bc}	0.043±0.88 ^{b}
10% Dried sardine fish	164.0±3.16 ^{a}	194.0±13.19 ^{cd}	32.6±12.08 ^{bcd}	13.02±0.55 ^{c}	0.045±1.08 ^{b}
20% Dried sardine fish	162.80±2.17 ^{a}	207.0±14.21 ^{bc}	44.2±14.16 ^{bc}	13.88±0.54 ^{b}	0.057±1.16 ^{b}

Mean with the same letters in the column are not significantly different at P<0.05

oInitial Body Weight, ° Final Body Weight, * Body weight Gain, † Feed Intake, ‡Feed Efficiency Ratio

Table 4: Effect of skimmed milk, soybean flour and dried sardine fish on weight of organs and weight right femur of osteoporotic female rats

Groups	Weight of liver (g)	Weight of kidney (g)	Weight of heart (g)	Weight of spleen (g)	Weight of right femur (g)
Control negative	4.66±0.513^{c}	1.18±0.130^{a}	0.680±2.41^{a}	0.680±0.730^{ab}	1.4433±0.362^{a}
Control positive	6.48±0.645^{a}	1.300±0.070^{a}	0.560±0.054^{a}	0.640±0.114^{a}	0.7696±0.034^{d}
10% Skimmed milk	5.86±1.12^{ab}	1.34±0.161^{a}	0.560±0.061^{a}	0.660±0.151^{ab}	0.9460±0.018^{c}
20% Skimmed milk	5.64±0.573^{ab}	1.42±0.327^{a}	0.660±0.089^{a}	0.760±0.134^{a}	1.3293±0.106^{a}
10% Soybean flour	5.40±0.469^{bc}	1.32±0.044^{a}	0.600±0.070^{a}	0.700±0.001^{ab}	0.8563±0.013^{cd}
20% Soybean flour	5.48±0.460^{bc}	1.22±0.363^{a}	0.520±0.044^{a}	0.600±0.070^{ab}	0.9176±0.005^{cd}
10% Dried sardine fish	5.58±0.342^{b}	1.28±0.083^{a}	0.600±0.070^{a}	0.720±0.130^{ab}	0.9273±0.013^{cd}
20% Dried sardine fish	5.44±0.585^{bc}	1.32±0.044^{a}	0.620±0.109^{a}	0.580±0.109^{b}	1.1643±0.061^{b}

Mean with the same letters in the column are not significantly different at P<0.05

in body weight gain. The decrease in body weight gain may be due to high dose from cortisone and long-term.

Effect of skimmed milk, soybean flour and sardine fish on weight organs are shown in Table 4. Results revealed that there are no significant differences in weight of kidney, heart and spleen between all groups. Meanwhile, positive control showed significant increase in weight of liver and decreases in weight of femur bone (6.48 and 0.7696 g) compared to the control negative (4.66 and 1.4433 g), injection with cortisone for 5 week caused increases in liver weight and decrease in weight of femur bone. These results approved by Gouda [55] and Stanley *et al.* [56], who reported that cortisone caused the cells of regeneration liver to be increased in size and weight through the increase infiltration of lipid and decreases in femur bone weight. Also, Gouda [55] reported that mean value ±SD of liver weight/body weight of positive control (rats administration cortisone) increased as compared to negative control group. However, Howard Clark and Leroy [57] reported that administration of cortisone to rats increased the protein content and relative weight of the liver of animal. Group of rats was administration diet supplemented with soybean flour at low and high concentration (10 and 20%) and sardine fish at high concentration (20%) lead to decrease weight of liver and showed no significant difference between this groups and negative control group. The decrease in liver weight of soybean flour may be due to soybean contain isoflavone and the decrease in liver weight of rats fed diet supplemented sardine fish may be due to omega-3 present in sardine fish (35.86%). These results were approved by Bamz *et al.* [58] and Leng *et al.* [59] who reported that rats injected with soybean isoflavones (genistein and daidzien) lower of liver weight than versus vehicle control rats. Also, Meganathan *et al.* [60], mentioned that rats administration omega-3 fatty acids decrease weight of liver (5.76 g) which increased (6.43 g) by treated with baracetamol. Andrea *et al.* [61], reported that omega-3 fatty acid have benefit effect of liver disease.

Effect of skimmed milk, soybean flour and sardine fish on bone marker of osteoporotic rats are shown in Table 5. The concentration mean of serum osteocalcin, a parameter of bone formation in positive control group was decreasing significantly $P<0.05$ (10.33ng/ml) in comparison to negative control group (27.30 ng/ml). Osteocalcin is primarily deposited in the extracellular matrix of bone, but a small amount enters the blood. Serum osteocalcin is sensitive and specific marker of osteoblastic activity and its serum level thus reflect the rate of bone formation [62]. All of treatment showed significant increase $P<0.05$ in serum osteocalcin concentration. The best results of osteocalcin concentration was observed in osteoporotic rats fed on skimmed milk at low (23.27 ng/ml) and high levels (26.60 ng/ml) and sardine fish at low (18.90 ng/ml) and high (20.30 ng/ml) concentration compared to control positive group (10.33 ng/ml). The osteoporotic rats feeding with diet supplemented with 20% skimmed milk showed no significant difference was observed with positive control group. The increased in osteocalcin may be due to skimmed milk and sardine fish have higher calcium content (2000 and 1940 mg/100 ml. These results are in agreement with Kruger *et al.* [63], who reported that supplementation the diet with skimmed milk high calcium for 16 week lead to increased total osteocalcin compared to control group received diet no supplementation. Data in Table 5 also showed decreased in estradiol E2 and increased in parathyroid hormone PTH of control positive (15 and 67.50 pg/ml) compared to control negative (27 and 34.73 pg/ml). Abaza *et al*. [64] reported serum E2 and PTH was significantly lower in the osteopenic group than osteoporotic group. Increased PTH recreation contributes to an increase in bone resorption and osteoporosis [65], over production of PTH lead to an increase in bone resorption compared with bone formation and contributes to general skeleta demineralization. However, rats feeding on skimmed milk, soybean flour and sardine fish revealed significantly increased in E2 and significantly decreased in PTH. The reduced in PTH in rats fed on diet supplemented with 20% skimmed milk was higher (represent 44.99%) compared to other groups. These results are in agreement with those obtained by Micheal *et al*. [66], who reported that supplementation diet with calcium showed a decrease in PTH.

Data in Table 6 illustrated that there are significantly decrease $p<0.05$ in serum calcium and phosphorus concentration in control positive (2.058 and 2.083 mmol/l) compared to negative control group (4.133 and 3.966 mmol/l). The decrease in calcium may be due to cortisone decrease calcium absorption from intestinal. Glucocorticoid affects mineral homeostasis by reducing calcium absorption and causing secondary hyper parathyrodism. Glucocorticoid directly inhibit osteoblastic bone formation, impair intestinal calcium excretion absorption and promote renal calcium excretion [67]. All treatment showed significant increase $p<0.05$ in serum calcium and phosphorus, the highest concentration of calcium was observed in serum rats fed on diet supplemented with 20% (skimmed milk, followed by

Table 5: Effect of skimmed milk, soybean flour and dried sardine fish on bone marker of osteoporotic female rats

Groups	Osteocalcin (ng/ml)	Parathyroid hormone (PTH) (pg/ml)	Estradiol (E2) (pg/ml)
Control negative	27.30±0.13 [a]	34.73±2.33 f	27.0±0.89 [a]
Control positive	10.33±0.67g	67.50±1.439 [a]	15.0±0.09 [e]
10% skimmed milk	23.27±1.28 [c]	57.56±1.90 [c]	19.33±2.25 [cd]
20% skimmed milk	26.60±1.61 [a]	37.13±3.70 [e]	28.33±3.14 [a]
10% Soybean flour	14.90±0.36 f	60.33±0.81 [b]	17.33±1.37 [d]
20% Soybean flour	18.90±0.36 [d]	51.10±0.94 [d]	20.0±1.79 [c]
10% Dried sardine fish	16.70±0.36[e]	59.66±1.72 [bc]	18.33±2.25 [cd]
20% Dried sardine fish	20.30±0.80 [c]	49.30±0.97[d]	22.33±1.37 [b]

Mean with the same letters in the column are not significantly different at P<0.05

Table 6: Effect of skimmed milk, soybean flour and dried sardine fish on calcium, phosphorus in serum and in bone of osteoporotic female rats

	Serum(mmol/l)		Bone (mg/100g) Femur	
Groups	Calcium	Phosphorus	Calcium	Phosphorus
Control negative	4.133±0.68 [a]	3.966±0.619 [a]	19.226±0.278 [a]	11.533±0.314 [a]
Control positive	2.058±0.354[c]	2.083±0.085 [d]	10.833±1.351[d]	6.816±0.473 [d]
10% Skimmed milk	3.147±0.83 [b]	2.687±0.248 [cd]	15.60±0.473 [bc]	9.70±1.23 [c]
20% Skimmed milk	4.117±0.62 [a]	3.0±0.107 [bc]	18.733±0.33 [a]	10.133±0.895[bc]
10% Soybean flour	2.766±0.089 [b]	2.533±0.125 [d]	15.700±0.558[bc]	9.733±1.19 [c]
20% Soybean flour	3.263±0.85[b]	2.767±0.089 [cd]	16.200±0.983[b]	10.220±0.232 [bc]
10% Dried sardine fish	2.943±0.309 [b]	3.237±1.89 [b]	15.166±0.900 [c]	10.760±0.374 [ab]
20% Dried sardine fish	3.263±0.98 [b]	3.973±0.135 [a]	18.733±0.338 [a]	11.366±0.232[a]

Mean with the same letters in the column are not significantly different at P<0.05

sardine fish and soybean flour). These results may be due to skimmed milk and sardine fish have higher calcium content and easy absorbed by body. Also, lactose present in skimmed milk increase calcium absorption. These results were approved by Napoli *et al.* [68], who reported that the best dietary of calcium is dairy products because of the favorable element calcium content and absorption ability of calcium was higher. Larson *et al.* [69] reported calcium from fish would be easily absorbed by body and the intake of small fish with bones could increase calcium bioavailability.

Data in Table 6 also showed a decrease in calcium and phosphorus femur bone (10.339 and 6.816 mg/100g) compared to negative control group (19.226 and 11.533 mg/100g). The decrease in calcium concentration may be due to the cortisone lead to limited calcium absorption by gastrointestinal. Also, its increase urinary excretion of calcium and decrease bone uptake of calcium. All treatment group with two concentrations (10% and 20%) showed significant increase in calcium and phosphorous femur bone as compared to positive control group. The best results of femur bone calcium and phosphorous recorded of osteoporotic rats fed diet supplemented with 20% skimmed milk followed by 20% sardine fish and 20% soybean flour. These results may be due to higher bioavailability of calcium and phosphorous in skimmed milk and sardine fish and deposition of them in bone; and present isoflavones in soybean flour. Calcium in dairy products has high bioavailability and thus shows greater absorption in the intestine than calcium from non dairy products, like dark green vegetable, broccolis and soybean are common dietary source of calcium, but the calcium they contain has low bioavailability [70,71]. These results are in line with the results obtained by Ragab and Abd-Elkhalek [72], they demonstrated that gensitein in soybean flour had effect high significantly on content of rats tibia and femur from calcium and phosphorous.

Total BMD and BMC of femur bone each group are summarized in Table 7. Results showed that osteoporosis caused significant decrease of BMD and BMC of femur bone. The Glucocorticoid has harmful effects on bone density [73]. Glucocorticoid induced osteoporosis and leads to a suppression of bone formation by decrease the number and function osteoblast and induced bone loss [74-76]. There are many reported to decrease BMD and BMC in osteoporosis disease [77, 78]. The mean BMD and BMC of osteoporotic rats fed diet supplemented with 20% skimmed milk and 20% sardine fish was higher (0.15573, 0.1096 and 0.14360, 0.10666 g/cm^2) than soybean flour (0.13953 and 0.10685 gm/cm^2). The increases in BMD and BMC due to increases calcium and phosphorus in diet lead to increases ostoblast cell which lead to increases in bone formation. These results are in

Table 7: Effect of skimmed milk, soybean flour and dried sardine fish on BMD and BMC of osteoporotic female rats

Groups	°BMD G/cm²	oBMC G/cm²
Control negative	0.16140±0.002^a^	0.10997±0.057^a^
Control positive	0.0810±0.011^d^	0.10267±0.034^d^
10% skimmed milk	0.12856±0.005^c^	0.10787±0.007^abc^
20% skimmed milk	0.15573±0.009^a^	0.10963±0.028^ab^
10% Soybean flour	0.12760±0.006^c^	0.10400±0.045^cd^
20% Soybean flour	0.13953±0.024^b^	0.10685±0.006^abc^
10% Dried sardine fish	0.12733±0.002^c^	0.10440±0.004^bcd^
20% Dried sardine fish	0.14360±0.002^b^	0.106667±0.001^abcd^

Mean with the same letters in the column are not significantly different at P<0.05 Boon Mineral Density, °Boon Mineral concentration *

Table 8: Effect of skimmed milk, soybean flour and dried sardine fish on AST, ALT and ALP of osteoporotic female rats

Groups	AST (U/L)* Mean± SD	°ALT (U/L) Mean± SD	†ALP (U/L) Mean± SD
Control negative	93.97±1.39^c^	45.33±2.25^f^	257.0±0.84^b^
Control positive	140.30±1.75^a^	63.66±2.87^a^	292.0±2.36 ^a^
10% skimmed milk	138.0±0.005^a^	61.33±4.59^ab^	289.0±0.98 ^b^
20% skimmed milk	132.33±0.728^a^	57.33±5.39^bc^	272.0±3.22^c^
10% Soybean flour	120.33±0.338^b^	55.22±1.36^cd^	268.33±1.37 ^d^
20% Soybean flour	113.66±1.86^b^	48.32±2.87^ef^	261.33±1.37^f^
10% Dried sardine fish	114.0±0.357^b^	51.36±2.73^df^	265.33±2.73^e^
20% Dried sardine fish	84.0±0.193^c^	47.23±4.03^ef^	259.0±2.36^fg^

Mean with the same letters in the column are not significantly different at P<0.05 *Aspartate amino transferees, °Alanine amino transferee, †Alkaline phosphates.

agreement with the findings of Rizzoli *et al.* [13] and Bakary *et al.* [79], they reported that increasing calcium intake or dairy products is associated with a greater gain in BMD and BMC. Zhu and Prince [14] reported those milk and calcium intakes are related to bone mineral accretion during growth. Farina *et al.* [80] reported that women with high intake of dark fish (Salmon) have protective effect of bone loss because the increase the intake of calcium and vitamin D, increasing the calcium intake preventing bone loss, possibly due to the effect of calcium in suppressing PTH secretion. Mugurma *et al.* [20] reported that diet supplemented with (soybean and skimmed milk combination) was enhanced bone mass density in Wister rats.

Effect skimmed milk, soybean flour and sardine fish on AST, ALT and ALP are shown in Table 8. Data in Table 8 revealed that there are significantly increase in AST, ALT and ALP in positive control (140.30, 63.66 and 292 U/L), respectively compared to control negative (93.97, 45.33 and 257 U/L), respectively. The increased in liver function may be due corticocoticoid therapy is associated with several forms of liver injury [56]. Women treated with 5ml corticosteroid therapy daily increased AST and ALT [81]. Glucocorticoids have adult effect on type1 collagen synthesis and ALP [74]. The levels of AST and ALT and ALP decreased gradually with increasing the sardine fish and soybean flour concentration. The decrease in AST, ALT and ALP in group supplemented with soybean flour may be due to isoflavone in soybean flour (Daidzein and Genistein) and the decrease in liver function of group was supplemented with sardine fish may be due to high content of omega-3 (35.86%) in sardine fish. These results are in agreement with the results of Heller *et al.* [82], who reported fish oil supplemented improved liver function. Gudbrandsen *et al.* [83] reported dietary supplementation of soy protein enrich isoflavones has been decreased in ALT and ALP.

Histopathological Results: Microscopically, bone of rat from negative control revealed no histopathology change (Photo 1). Meanwhile, section of bone of rat from positive control group (rats suffering from osteoporosis) revealed thin bone cortex and dilatation bone marrow cavity (Photo 2); cracks and necrosis in bone cortex (Photo 3). This results agreement with Gouda [55], reported that the rats treated with cortisone showed reduced bone mass and reduced in thickness of cortical bone. The bone of rats from group supplemented with 10% skimmed milk showed normal bone cortex (Photo 4). Moreover, the bone of rats from group supplemented 20% skimmed milk revealed no histopathological changes except thick cortical bone (Photo 5) and proliferation of osteoblasts (Photo 6). These results are in line with the results obtained by Rizzoli [13], who reported Chinese children receiving milk supplements increased cortical bone thickness. Meanwhile, bone of rat from group supplemented by 10% soybean flour showed thick cortical bone (Photo 7) whereas, few section revealed cracks in cortical bone. Section from group supplemented 20% soybean flour should thick cortical bone (Photo 8). Thick cortical bone was noticed in bone rat from group supplemented with 10% sardine fish showed thick of cortical bone photo (9), whereas section of rat from group supplemented with 20% sardine showed very thick cortical bone (Photo 10).

Microscopically, liver of rats from negative control group revealed no histological change (Photo 11). Section from positive control group should cytoplasmic vacuolization of hepatocytes (Photo 12) and necrosis of sporadic hepatocytes (Photo 13). These results may be due to cortisone induce changed in histological structure of liver. These results are agreement with the results by Gouda [55], who reported that cortisone caused cutoplasmic vacuolization of hepatocytes and sinusoidal

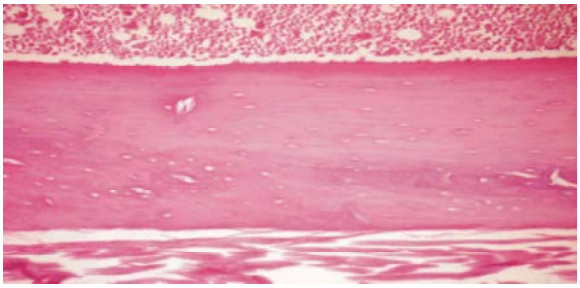

Photo 1: Photomicrograph of bone of rat from negative control group showing no histopathology changes (H and E x 200).

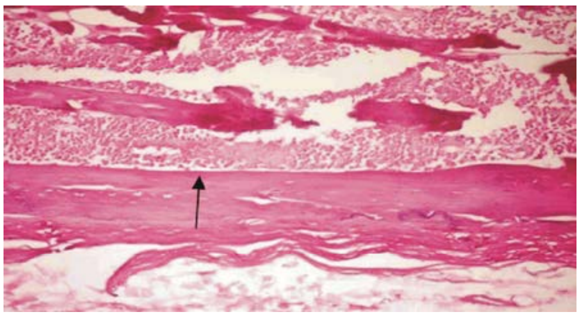

Photo 2: Photomicrograph of bone of rats from positive control group showing thin bone cortex and dilatation of bone marrow vavity (H and E x 200).

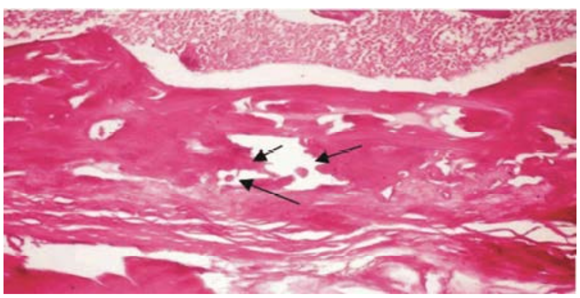

Photo 3: Photomicrograph of bone of rats from positive control group showing cracks and necrosis in bony cortex (H and E x 200).

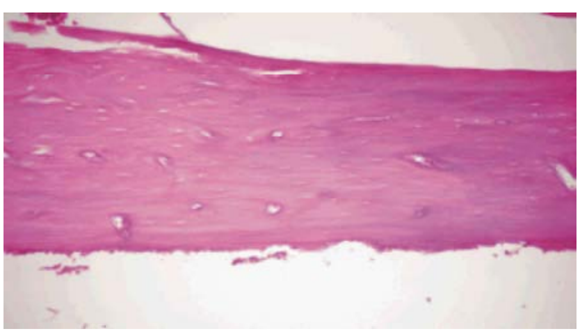

Photo 4: Photomicrograph of bone rats from group supplemented with 10% skimmed milk showing normal bone cortex (H and E x 200).

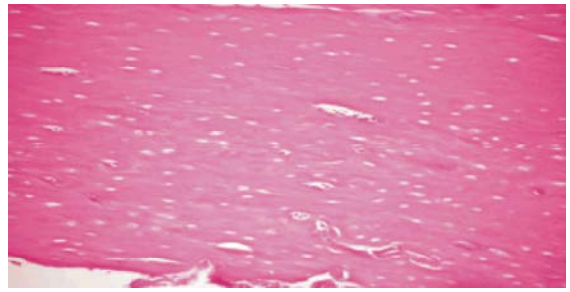

Photo 5: Photomicrograph of bone of rats from group supplemented with 20% skimmed milk showing thick cortical bone (H and E x 200).

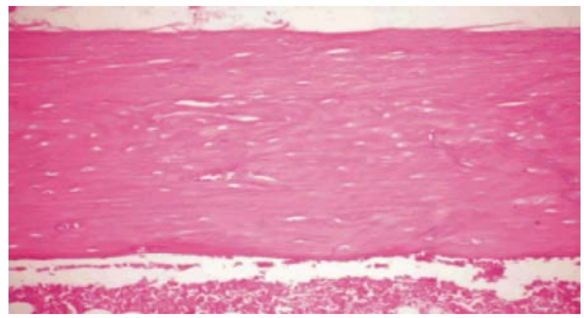

Photo 6: Photomicrograph of bone of rats from group supplemented with 20% skimmed milk showing proliferation of osteoblasts (H and E x 200).

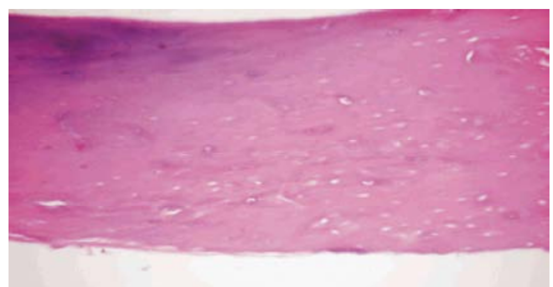

Photo 7: Photomicrograph of bone rats from group supplemented with 10% soy flour showing thick cortical bone (H and E x 200).

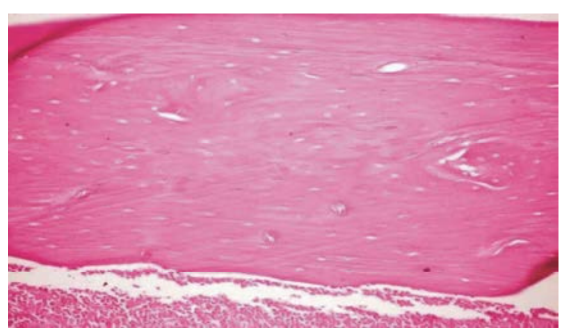

Photo 8: Photomicrograph of bone rats from group supplemented with 20% soy flour showing thick cortical bone (H and E x 200).

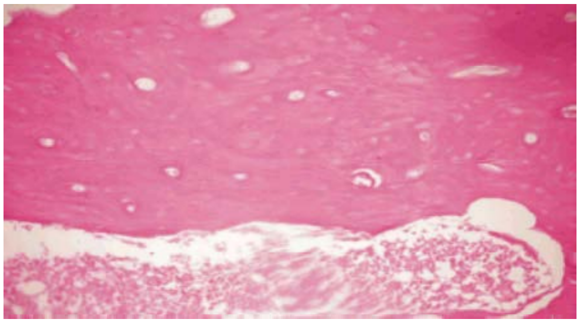

Photo 9: Photomicrograph of bone rats from group supplemented with 10% sardine fish showing thick cortical bone (H and E 200).

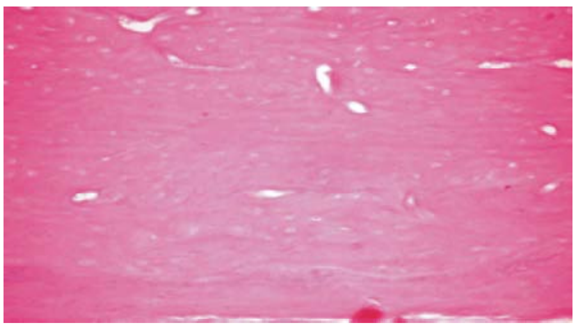

Photo 10: Photomicrograph of bone rats from group supplemented with 20% sardine fish showing very thick cortical bone (H and E x 200).

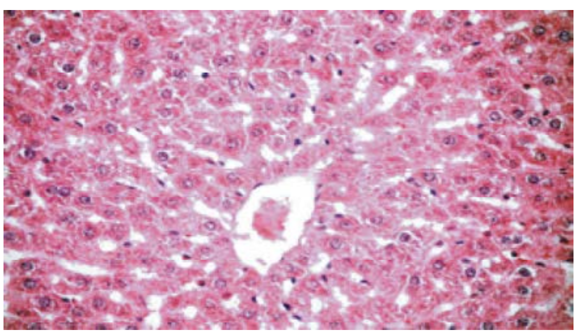

Photo 11: Liver of rat from control negative showing no histological change (H and E x 400).

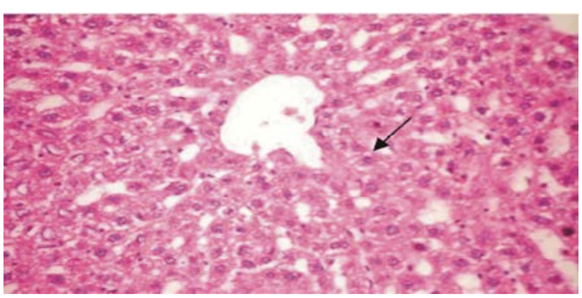

Photo 12: Liver of positive control group showing cytoplasmic vacuolization of hepatocytes (H and E x 400).

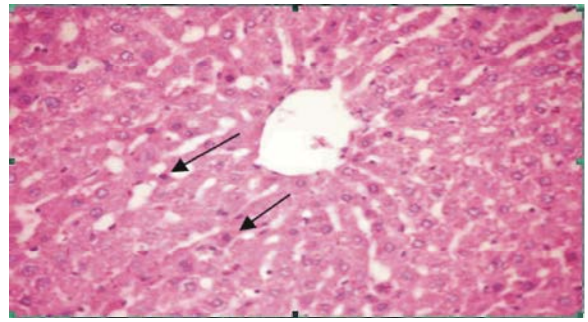

Photo 13: Liver of rat from positive control group showing necrosis of sporadic hepatocytes (H and E x 400).

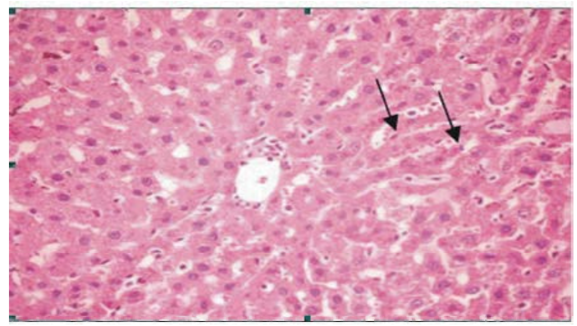

Photo 14: Liver of rat from group supplemented with 10% skimmed milk showing kuffer cells activation, congestion of hepatic simusoids and vacuoles in the cytoplasm hepatocytes (H and E x 400).

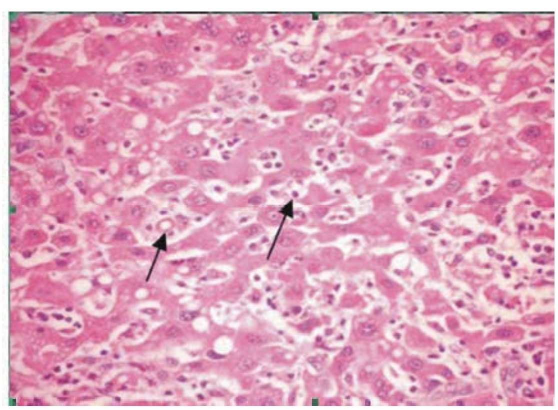

Photo 15: Liver of rats for group supplemented with 20% skimmed milk showing fatty degeneration of hepatocytes and massive inflammatory cells infiltration (H and E x400).

leucocytosis of liver. However, liver of rats from group supplemented 10% skimmed milk revealed kupffer cells activation, congestion of hepatic sinusoids and vacuoles in the cytoplasm hepatocytes (Photo 14). Meanwhile,

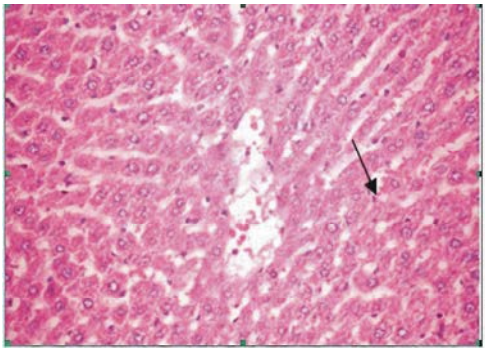

Photo 16: Liver of rat from group supplemented 10% soy flour showing slight activation of kupffer cells (H and E x 400)

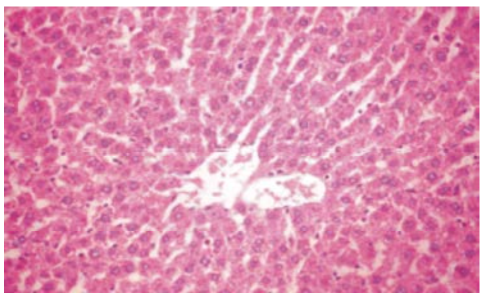

Photo 17: Liver of rats from group supplemented with 20% soy flour showing no histopathological changes (H and E x 400).

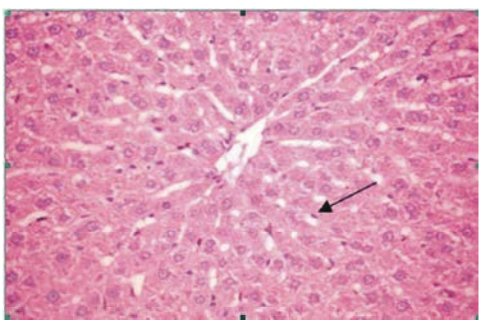

Photo 18: Liver of rats from group supplemented with 10% sardine fish showing slight activation of kupffer cell (H and E x400).

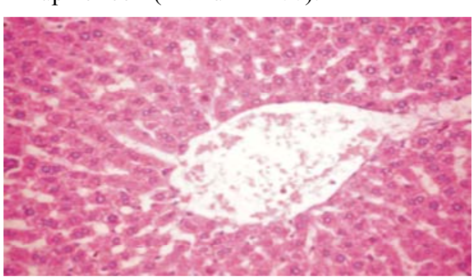

Photo 19: Liver of rats from group supplemented with 20% sardine fish showing no histopathological changes (H and E x 400).

liver of rat from group supplemented 20% skimmed milk should fatty degeneration of hepatocytes and massive inflammatory cells infiltration (Photo 15). Liver of rat from group supplemented 10% soybean flour should slight activation of kupffer cells (Photo 16), whereas, section from group supplemented with 20% soy flour no histopathological changes (Photo 17). Meanwhile, liver of rats from group supplemented with 10% sardine fish slight activation of kupffer (Photo 18). Moreover, liver of rats from group supplemented 20% sardine fish should no histopathological change accept slight activation of kupffer cells (Photo 19).

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